Point Mutations Within and Outside the Homeodomain Identify Sequences Required for *proboscipedia* Homeotic Function in Drosophila

Corinne Benassayag,* Muriel Boube,* Laurent Seroude† and David L. Cribbs*

**Centre de Biologie du Développement-CNRS, 31062 Toulouse Cedex, France and †Division of Biology, California Institute of Technology, Pasadena, California 91125*

Manuscript received January 29, 1997
Accepted for publication March 21, 1997

ABSTRACT

The Drosophila homeotic gene *proboscipedia* (*pb*) encodes a homeodomain protein homologous to vertebrate HoxA2/B2 required for adult mouthparts formation. A transgenic Hsp70-pb (HSPB) element that rescues *pb* mutations also induces the dominant transformation of antennae to maxillary palps. To identify sequences essential to PB protein function, we screened for EMS-induced HSPB mutations leading to phenotypic reversion of the HSPB transformation. Ten revertants harbor identified point mutations in HSPB coding sequences. The point mutations that remove all detectable phenotypes *in vivo* reside either within the homeodomain or, more unexpectedly, in evolutionarily nonconserved regions outside the homeodomain. Two independent homeodomain mutations that change the highly conserved Arginine-5 in the N-terminal hinge show effects on adult eye development, suggesting a previously unsuspected role for Arg5 in functional specificity. Three additional revertant mutations outside the homeodomain reduce but do not abolish PB^+ activity, identifying protein elements that contribute quantitatively to *pb* function. This *in vivo* analysis shows that apart from the conserved motifs of PB, other elements throughout the protein make important contributions to homeotic function.

THE repetitive segments of *Drosophila melanogaster* established in early embryogenesis come to differ from each other due to the action of a small set of homeotic selector genes. These genes attribute unique developmental fates to segment primordia, then maintain these identities throughout development (GARCIA-BELLIDO 1975, 1977; LEWIS 1978). The general biological importance of these genes is apparent in their remarkable conservation across the animal kingdom from nematodes to humans (MCGINNIS and KRUMLAUF 1992; KENYON 1994). Dramatic functional demonstrations of their developmental roles have been obtained in ectopic expression assays, where homeotic selector proteins can induce the development of normal structures in novel positions (SCHNEUWLY *et al.* 1987; KUZIORA and MCGINNIS 1988; GIBSON *et al.* 1990; MALICKI *et al.* 1990; MANN and HOGNESS 1990; GONZALEZ-REYES *et al.* 1992; HEUER and KAUFMAN 1992; LAMKA *et al.* 1992; CRIBBS *et al.* 1995; HALDER *et al.* 1995).

Eight Drosophila homeotic loci are clustered in the Bithorax and Antennapedia Complexes (PEIFER *et al.* 1987; KAUFMAN *et al.* 1990; MCGINNIS and KRUMLAUF 1992). These HOM genes encode nuclear proteins possessing the 60-amino acid homeodomain motif that contributes to sequence-specific DNA binding and confer segmental identity through the transcriptional activation or repression of downstream genes (GARCIA-BELLIDO 1977; GEHRING *et al.* 1990; HAYASHI and SCOTT 1990; ANDREW and SCOTT 1992; BOTAS 1993). The growing number of identified targets comprises genes that encode regulatory molecules such as the Drosophila TGF-β growth factor homologue *decapentaplegic* (CAPOVILLA *et al.* 1994), the transcription factor *Distal-less* (VACHON *et al.* 1992; O'HARA *et al.* 1993), and structural molecules such as *connectin* (GOULD and WHITE 1992; NOSE *et al.* 1992) and *centrosomin* (HEUER *et al.* 1995; LI and KAUFMAN 1996).

Most HOM protein homeodomains bind the same or closely related sequences with similar affinities *in vitro* (EKKER *et al.* 1991, 1992, 1994), but these similarities poorly reflect the very different developmental programs induced *in vivo*. Target genes might be sensitive to subtle differences in DNA binding by different homeoproteins *in vivo*, for example via cooperative DNA binding as detected for the homeotic Ultrabithorax protein (BEACHY *et al.* 1993). Alternatively, small differences in affinity might be amplified by interactions of the homeoprotein with protein cofactors. One cofactor identified to date is the *extradenticle* (*exd*) gene, whose highly conserved EXD protein product acts in combination with the homeotic genes to help specify distinct developmental fates (PEIFER and WIESCHAUS 1990; RAUSKOLB *et al.* 1993, 1995; GONZALEZ-CRESPO and MORATA 1995). This partnership occurs at least in part through combinatorial DNA binding (CHAN *et al.* 1994; VAN DIJK and MURRE 1994; JOHNSON *et al.* 1995).

To examine how HOM proteins achieve their specific functions *in vivo*, one fruitful approach has been to

Corresponding author: David Cribbs, CNRS, Centre de Biologie du Développement, 118 route de Narbonne, Bâtiment 4R3, 31062 Toulouse Cedex, France. E-mail: cribbs@cict.fr

study the properties of modified HOM proteins. Studies using chimeric HOM proteins have shown that much of the specificity resides in the homeodomain and a small number of nearby residues, including sequences immediately downstream of the homeodomain and the YPWM motif involved in specific interactions with the cofactor EXD (GIBSON *et al.* 1990; MANN and HOGNESS 1990; CHAN and MANN 1993; ZENG *et al.* 1993; JOHNSON *et al.* 1995; MANN 1995; ZHAO *et al.* 1996). However, several studies have reported that homeodomain DNA binding activity is neither strictly required nor sufficient for some homeodomain protein functions *in vivo* (ANANTHAN *et al.* 1993). For example, the Drosophila segmentation gene *fushi tarazu* (*ftz*) encodes a homeodomain containing protein that can act as a direct DNA binding transcriptional regulator *in vivo* (SCHIER and GEHRING 1992). A FTZ polypeptide rendered incapable of DNA binding by removing its homeodomain can still regulate *ftz* expression *in vivo* and can still control expression of its target gene *wingless* through direct interactions with the pair-rule protein Paired (FITZPATRICK *et al.* 1992; COPELAND *et al.* 1996). Taken together, these results suggest that DNA binding and protein-protein interactions involved in homeoprotein function may involve not only the homeodomain and adjacent regions but also other domains along the protein.

In the present work we wished to study homeotic structure-function relationships for the Drosophila *proboscipedia* (*pb*) gene. This locus codes for a protein strongly conserved in mice and humans [for example, the three homeodomains are 95% identical (CRIBBS *et al.* 1992)]. *pb* function is required for the correct development of the labial and maxillary palps composing the adult mouthparts. The *pb* gene is unique among the known homeotic loci in two respects. First, it appears not to be required for embryonic development despite its prominent localized early expression (PULTZ *et al.* 1988). Indeed, *pb* animals eclose as adults with labial palps and maxillary palps transformed toward prothoracic leg and antennal identities, respectively (KAUFMAN 1978). Second, *pb* function is dose sensitive. Partial loss-of-function mutations lead to a distinct transformation of the labial palps to antennal aristae instead of prothoracic legs (KAUFMAN 1978; PULTZ *et al.* 1988). Inversely, ectopic PB expression in transgenic Hsp70:pb (HSPB) flies directs a fully penetrant and dose-sensitive dominant transformation of antennae toward maxillary palps. The level of pb^+ function present in a segment is central to determining its identity since different levels of PB function or protein expression can give rise to distinct developmental outcomes (CRIBBS *et al.* 1995).

To better define PB protein structural elements important to its *in vivo* function, we have performed a novel screen to isolate point mutations that abolish the dominant HSPB antennal transformation. The genetic and molecular analysis of these mutants reinforce the notion that the homeodomain plays a preponderant role in homeotic function. However, we show that point mutations in nonconserved regions can also abolish *in vivo* PB homeotic function as completely as a mutation within the homeodomain. Our analysis has also identified protein elements situated outside the homeodomain in the C-terminal and N-terminal portions of the protein that contribute quantitatively to *pb* function with qualitative developmental consequences *in vivo*.

MATERIALS AND METHODS

Fly culture and phenotypic analysis: Stocks were cultured on standard cornmeal/agar medium at 22° unless otherwise indicated. Phenotypes were initially examined under a stereomicroscope; detailed analyses were performed by scanning electron microscopy or by light microscopy (Zeiss Axiophot) after mounting in Hoyer's medium.

The transgenic strain used for the screen, HSPB:2-5, has been described previously (CRIBBS *et al.* 1995). The *pb4* allele is a partial loss-of-function mutation truncated for its C-terminal region (CRIBBS *et al.* 1992).

Phenotypic reversion analysis: Male HSPB:2-5 flies were mutagenized with 25 mM EMS overnight (LEWIS and BACHER 1968) then mated to w^{1118} (henceforth, *w*) virgin females. After 4 days the males were removed to ensure that only post-meiotic events were recovered. To verify the presence of the HSPB element, only females with orange eyes (mini-w^+) were examined for reversion of the antenna-to-maxillary palp transformation. Candidate females were recrossed with w males, and HSPB-bearing progeny with normal antennae were kept as revertants. Due to multiple mutagenic events on the *X* chromosome of interest, only female progeny with colored eyes were initially obtained in most cases. Continued outcrosses with *w* males yielded viable and fertile males with colored eyes (hence *X* chromosomes lacking accessory lethal mutations), permitting pure revertant lines to be established. To examine the phenotypic effects of mutant PB (PB*) overexpression, heat shocks were administered to embryos or larvae by placing the culture tubes in a 35° water bath for 3 hr. This treatment was repeated up to four times.

Testing rescue of *pb* mutations by HSPB revertant lines: To test the rescuing capacity of the revertant elements for the pb^4/pb^4 mutant, females carrying wild-type or mutated HSPB elements were crossed with *w; Ki* pb^4 p^p/TM6B, *Hu Tb* males. Male progeny carrying both the wild-type or mutated HSPB element and the pb^4 chromosome were recrossed with *w; Ki* pb^4 p^p/TM6B, *Hu Tb* females. All female progeny carry the HSPB element and of these one-fourth are homozygous for pb^4 (*w* HSPB*/*w; Ki* pb^4 p^p/*Ki* pb^4 p^p). We then examined the *pb*-induced homeotic transformation in the mouthparts of these females. To test for possible dosage effects on the rescue, we also crossed *w* HSPB*; *Ki* pb^4 p^p/TM6B, *Hu Tb* males with *w* HSPB*/*w; Ki* pb^4 p^p/TM6B, *Hu Tb* virgin females, both obtained in the F_1 of the previous cross. This allowed us to examine pb^4 males hemizygous for the HSPB* element (compared with heterozygous females, above) and pb^4 females homozygous for HSPB*.

Immunolocalizations: Antibodies and staining reactions were essentially as described (RANDAZZO *et al.* 1991). Heat shocks were administered to nonstaged embryos on 35 mm collection plates in an incubator (3 hr/35°). The temperature of the incubator was found to be crucial for the HSPB element (containing a mini-gene with seven introns, and not a pre-

TABLE 1

Oligonucleotide sequence and location within pb

Oligonucleotide	Sequence	nt position[a]
LS10	5′ GTTCCCAAGCTGAGGAATATCTAATACGC 3′	Exon 2: 21–49
LS4	5′ CAAATTGACATGGTCGATCGC 3′	Exon 2: 717–697
LS11	5′ TCCGCGCTCCGTTTATCACAGTTCGGGG 3′	Exon 2: 359–331
pbr3	5′ ATTCGCAGCCGTCGATGGCTGA 3′	Exon 2: 401–422
pb4	5′ TCCGCCAATGGCGCCGGATCCAA 3′	Exon 2: 492–470
I3	5′ CATTCACTCCGCAAGTCTCAAACG 3′	Intron 3[b]
LS1	5′ GGAATTCCGCTGCCGTGTGCG 3′	Intron 4[b]
I4	5′ GCACACACCTGGTCGGAACTC 3′	Intron 4[b]
pb3	5′ TGTTGTTGTTGTGACCCCGGGAA 3′	Exon 6: 860–837
pbr1	5′ GAACTGCCATCCGATGATATACC 3′	Exon 6: 799–821
pb6	5′ GGGACTAGGATAATAGCCTG 3′	Exon 8: 1639–1620
LS26	5′ CCGCCTCAAATGTGATCAGCGCCG 3′	Exon 7: 1019–1042
E85′	5′ ATGCACCCACCAGCAAGGCG 3′	Exon 8: 1488–1507
LS5	5′ TGGTGGTTAATCGCCGCTGGC 3′	Exon 8: 2109–2089
pb6	5′ GGGACTAGGATAATAGCCTG 3′	Exon 8: 1639–1620
pbE8	5′ CGAATTCGCCGCCTCGCCGCAC 3′	Exon 8: 1734–1755
LS6	5′ GCACATGCATCACCTGGGAAATGGGG 3′	Exon 9: 37–62
pb2	5′ ATTCCGGCGCAAAGTCGTTGGC 3′	Exon 9: 466–445
E9	5′ TTCCGAATGCCTGGCCCTGAAC 3′	Exon 9: 359–338
LS29	5′ GGCATGCGCCTGAGCCGGC 3′	Exon 9: 235–217

[a] Figure 3, B–D (CRIBBS *et al.* 1992).
[b] Not shown in CRIBBS *et al.* (1992).

spliced cDNA), a variation of 0.5° changing the pattern of PB expression detected from the Hsp70 promoter.

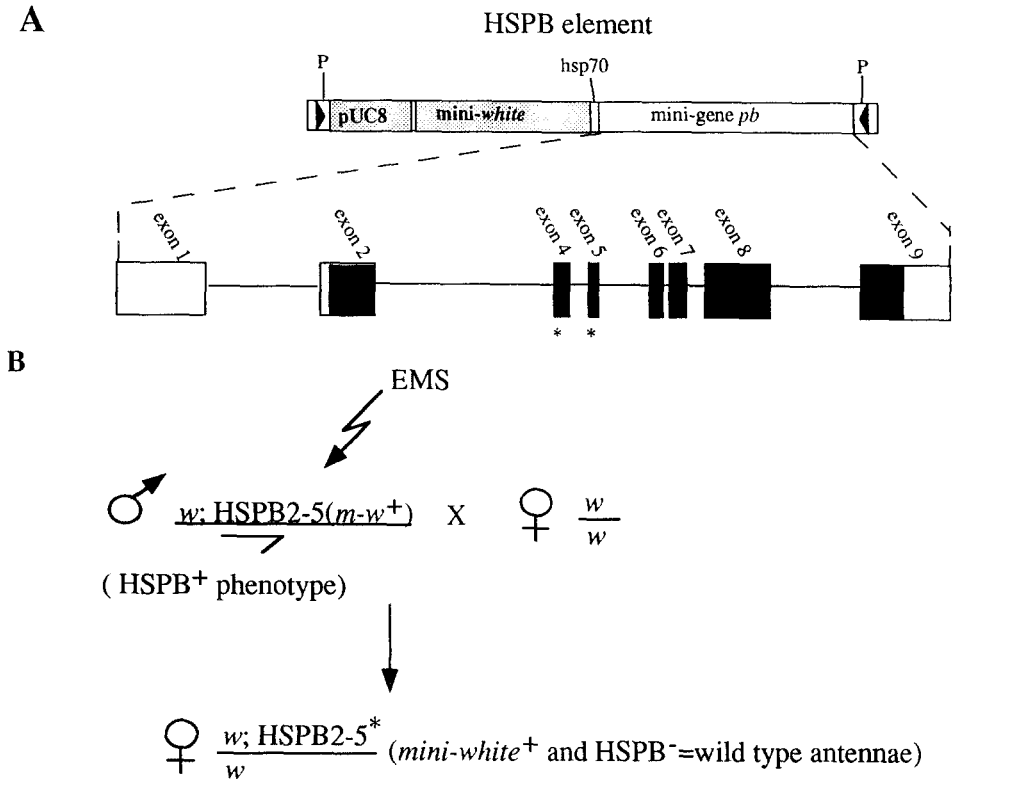
Characterization of the HSPB lesions: To prepare genomic DNA, 12 flies from each HSPB revertant line were homogenized into 250 μl of HB medium [0.2 M sucrose, 0.1 M Tris (pH 9.2), 50 mM EDTA, 0.5% SDS] then incubated at 68° for 10 min. Two hundred fifty microliters of TE [10 mM Tris HCl (pH 7.5)/0.1 mM EDTA] containing 0.5% SDS and 100 μg/ml proteinase K were added and incubated at 37° for 1 hr, then at 50° for 30 min. After adding 1.25 M potassium acetate followed by incubation at 4° for 10 min and centrifugation, DNA was extracted three times with phenol, phenol/chloroform and chloroform, then precipitated from a 0.3 M sodium acetate solution with 2 volumes of ethanol. DNA precipitates were washed with 70% ethanol then dissolved in 40 μl of TE. Typically 1 μl was sufficient for a 50 μl PCR reaction. Single base pair changes were detected by testing for hydroxylamine sensitivity as described by MONTANDON *et al.* (1989). Using this approach, it was possible to localize most of the HSPB revertants. Sequences of the located mutations were determined directly following asymmetric amplification of appropriate PCR product (MCCABE 1990) using the oligonucleotides listed in Table 1.

To ensure the accuracy of this approach, we sequenced the entire PB coding sequence of the characterized mutants. Double-stranded sequence was obtained for all putative mutations presented here. Double-stranded sequence was likewise obtained for the homeodomain of all lines. In four cases ($HSPB^{Rev3}$, $HSPB^{Rev14}$, $HSPB^{Rev17}$ and $HSPB^{Rev20}$) the transgenic allele was cloned in lambda EMBL3. For the others, the PCR amplification products contained a mix of the endogenous and transgenic sequences. To ensure that the mutation resides on the transgene and is not a simple polymorphism of the endogenous copy, parallel sequence runs were systematically carried out on the same gels for DNA from w^{1118} the parental HSPB:2-5 line and the HSPB plasmid injected to yield the transgenic lines. No polymorphisms were located within genomic protein-coding sequences.

RESULTS

Screen for HSPB phenotypic revertants: We performed a structure-function analysis of the homeotic gene *proboscipedia* (*pb*) to identify as precisely as possible structural elements contributing to pb^+ protein function. Several previous studies have addressed the question of structure/function relationships for homeotic proteins, by the construction of transgenic lines expressing internally deleted and/or chimeric homeotic proteins. Function is then assessed following heat-induced ectopic expression. Our strategy differed from those previously used in three respects. First, to minimize variability we employed the single representative transgenic line HSPB:2-5 line, which induces a fully penetrant, dose-sensitive dominant transformation of antennae toward maxillary palps without heat induction (Figure 2Ab). Second, we chose as mutagen the alkylating agent EMS that provokes primarily point mutations (G to A, and C to T), thus introducing minimal changes at random *in vivo*. Third, the new presumptive point mutations were selected, rather than constructed, as phenotypic revertant mutations that annul the developmental effects of $HSPB^+$. Newly isolated mutant stocks were characterized genetically for functional properties and molecularly to identify the new lesion.

The strategy of the reversion screen is described in Figure 1B. Males carrying the HSPB:2-5 element (see Figure 1A) were mutagenized with EMS and crossed with virgin *w* females. Female progeny with orange eyes (P[mini-$white^+$]) were screened in search of revertant females with wild-type antennae. Such revertant lines



C

Number of chromosomes screened	Number of revertant lines	Reversion frequency
50,000	21	4.2×10^{-4}

FIGURE 1.—Schematic representation of the HSPB element, the reversion screen and result. (A) The HSPB element (CRIBBS *et al.* 1995) comprises a visible marker, the mini-*white* gene conferring color to the eyes. The element also contains a *pb* mini-gene with complete PB coding sequences in exons 2–8 (■; noncoding RNA sequences are indicated in white) transcriptional fused to the Hsp70 promoter. The homeodomain is located in exons 4 and 5, indicated by asterisks. (B) HSPB:2-5 males with a dominant phenotype of transformation of the antennae to maxillary palps were mutagenized with EMS then mated to w^{1118} females. F_1 progeny were screened and females presenting a reversion of the HSPB phenotype with orange eyes (mini-w^+) were selected. (C) Among the 50,000 F_1 progeny screened, 21 mutations were isolated and established as independent lines.

should carry either a nonfunctional HSPB element, or a second-site suppressor of HSPB$^+$. Since the dominant antennal phenotype is obtained without heat induction, we anticipated that most of the mutations would reside within protein coding sequences of the HSPB element and not in the heat shock response pathway itself. However, this screen could in principle also yield other classes of recoverable mutations, notably dominant second-site modifiers. From ~50,000 mutagenized chromosomes screened, we obtained 21 revertant candidates (Figure 1C). For all 21 revertant lines, the revertant phenotype was fully penetrant and has not in any case been separated from the HSPB mini-w^+ marker. This indicates that the newly induced mutations are all located very near to or within the HSPB element.

Phenotypic characterization of the different revertant lines: Adult phenotypes were examined for each established revertant line with one or two copies of the HSPB^{Rev} element or after a heat induction. We also tested the capability of the HSPB revertant lines to rescue a partial *pb* loss of function mutation (pb^4 allele). We previously showed that this *pb* mutation, which leads to a reliable partial transformation of the labial palps to antennal aristae (Figure 2Ag), can be fully rescued by a single wild-type HSPB:2-5 transgene copy (Figure 2Ah). Assuming that the observed dominant homeotic transformation in the antennae reflects PB$^+$ function, reversion of PB function in the antennae should be paralleled by a loss of rescuing activity in the mouthparts.

The collection of revertant lines can be divided into three classes (Table 2). The first class contains apparent *pb* "null" mutations that abolish all HSPB effects detected in the antennae, mouthparts, wings, legs, and eyes (CRIBBS *et al.* 1995). This class comprises 14 lines, called HSPB$^-$. Such flies possess wild-type antennae in all tested conditions with no additional dominant phenotypes (Figure 2Ae). Similarly, none of these lines detectably rescues the pb^4 mutation with one or two transgene copies (Figure 2Ak). We should note that these revertant flies, which still contain the P insertion in the original site, are much more active and agile than the starting HSPB:2-5 line. This observation suggests that the reversion event has also abolished detrimental effects of PB in the nervous system.

A second class of HSPB revertant contained two lines (HSPB^{Rev17} and HSPB^{Rev3}) that behaved in a fashion qualitatively different from the others (Table 2). These lines showed a complete reversion of the dominant

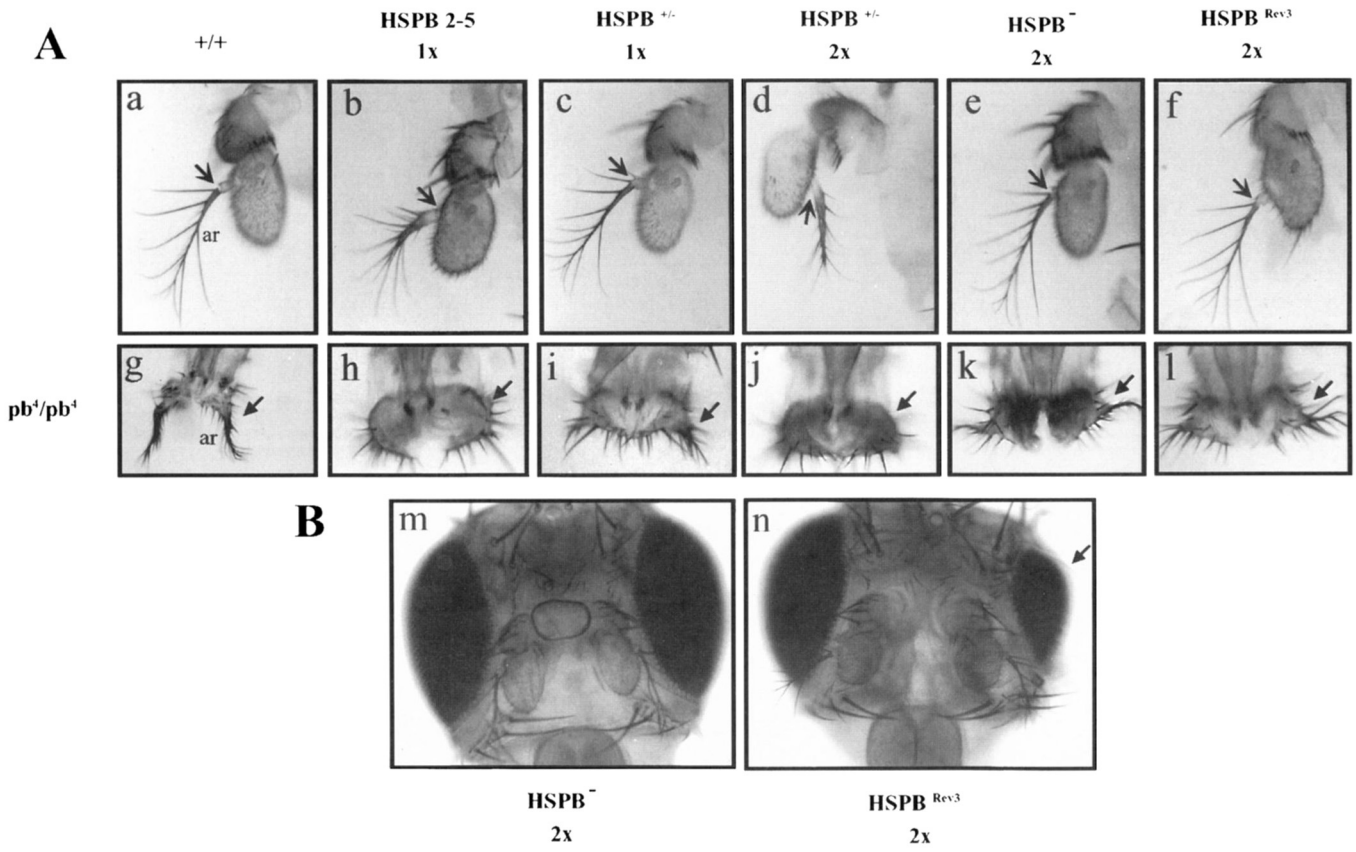


FIGURE 2.—Adult phenotypes of the different revertant classes. (A) Shown are antennal phenotypes (a–f) and rescue of the partial loss-of-function pb^4 mutation (g–l). (a) Wild-type antenna comprising, from proximal to distal, the antennal segments A1 (top right), A2, A3 and the plumed distal arista (ar: indicated with arrow). (b) HSPB:2-5 (one copy) antenna showing a partial antenna to maxillary palp transformation. Note the arista (arrow), which is shorter and thicker than for wild type. (c) Partial revertant line ($HSPB^{+/-}$, one copy of the mutated element). The arista is comparable to wild type (arrow; compare to a). (d) $HSPB^{+/-}$ revertant carrying two copies of the element. The transformation of the antenna to maxillary palps is comparable to HSPB:2-5 (in b), indicating a roughly twofold reduction in activity. (e) A fly carrying two copies of an $HSPB^{-}$ element with wild-type antenna. (f) Antenna of the $HSPB^{Rev3}$ line (third revertant class) showing a nearly wild-type arista (arrow, but note also the ectopic fine bristle on the bottom of A3). (g–l) Rescue of the hypomorphic pb^4 allele by the different revertant lines. (g) Distal labial palps of a pb^4 fly showing a partial transformation to arista (ar, indicated with arrow). (h) Labial palps of an HSPB:2-5;pb^4/pb^4 male showing complete rescue to wild-type labial palps (arrow). (i–l) Rescue of pb^4 labial palps by the different revertant classes. (i) $HSPB^{+/-}$;pb^4/pb^4. The transformation of the labial palps to arista is partial (arrow). (j) $HSPB^{+/-}$/ $HSPB^{+/-}$;pb^4/pb^4. The rescue of the transformation is complete. (k) $HSPB^{-}/HSPB^{-}$;pb^4/pb^4. No rescue of the labium to arista transformation is observed (arrow). (l) $HSPB^{Rev3}/HSPB^{Rev3}$;pb^4/pb^4. Little or no rescue of the labium to arista transformation is detected (arrow). (B) The eyes of an $HSPB^{-}$ homozygote ($HSPB^{Rev14}$; see m) are compared to those of an $HSPB^{Rev3}$ homozygote. The latter showed frequent but variable reductions (n).

ant → max transformation in a single copy, whereas one of the lines showed a slight but novel defect in two copies (appearance of a single, fine bristle on antennal segment 3; Figure 2Af). Following heat induction both lines showed detectable reductions of the adult eye. This reduction was weak for $HSPB^{Rev17}$ (not shown), whereas the eyes of $HSPB^{Rev3}$ flies were often markedly reduced (Figure 2Bn). These mutations remove *pb* homeotic activity in the mouthparts (Figure 2Al) and antennae but show a novel eye loss phenotype.

Revertant lines of the third class act like partial loss of function mutations. This class comprises three lines, called $HSPB^{+/-}$ (Table 2), that exhibit wild-type antennae with one $HSPB^{+/-}$ copy (Figure 2Ac), which is in the conditions used to select the revertant. However, these lines show a restored transformation of the antennae toward maxillary palps with two $HSPB^{+/-}$ copies similar to the transformation induced by one copy of the original HSPB element (compare Figure 2, Ad to Ab). Futhermore, two copies of these $HSPB^{+/-}$ elements suffice to detectably rescue a *pb* phenotype (Figure 2Aj) whereas a single copy shows partial rescue (Figure 2Ai). The dominant phenotypes and the partial rescues provided by the three lines are comparable. These mutations appear to affect quantitative aspects of PB function, modifying the number of HSPB copies required to give a threshold homeotic transformation.

Two additional HSPB revertant lines associated with lethal mutations or chromosomal rearrangements have not been established as homozygous stocks. Both lines

TABLE 2

Different classes of revertant phenotype

Classes	Name	No. of mutants	Phenotype in one copy	Phenotype in two copies	Rescue of pb^4 mutation
1	HSPB	14	Wild type	Wild type	−
2	$HSPB^{Rev3}$	1	Wild type	Antennas with a fine bristle on the A3 segment; eye reduction	−
2	$HSPB^{Rev17}$	1	Wild type	Wild type antennas; mild eye alteration	−
3	$HSPB^{+/-}$	3	Wild type	Transformation of antennas toward maxillary palps	+/−
Nonclassified	$HSPB^{Rev7\&13}$	2	Wild type	Lethal	ND

ND, not determined; +/−, partial rescue; −, a non-rescue.

exhibit complete reversion of the dominant antennal phenotype, and no rescue of a *pb* mutation, in the heterozygous condition (see Table 2).

Molecular characterization of mutations associated with $HSPB^{Rev}$ elements: To begin the molecular analysis, all 21 revertant lines were examined for PB protein accumulation in embryos after heat induction. We employed a polyclonal antiserum directed against the central portion of the protein including part of the homeodomain (CRIBBS *et al.* 1992). Revertant lines showing levels of ubiquitous PB protein accumulation similar to HSPB:2-5 after heat induction (17 of the 21 lines) were retained for molecular analysis. In these cases, the Hsp70 promoter remains functional and expresses at least the homeodomain-containing N terminal half of the PB protein.

We next sought to locate the new lesion and determine the specific sequence change, presuming that the close genetic linkage reflects mutation of PB coding sequences. Southern blot analysis revealed no detectable deletions or rearrangements in these 17 revertant lines (data not shown). Coding sequences of the 8.6 kb *pb* mini-gene were then examined to detect mismatched cytosine residues based upon their sensitivity to modification by hydroxylamine (MONTANDON *et al.* 1989). This method detects all base changes except A to T and T to A tranversions, and we therefore anticipated being able to detect the majority of EMS-induced mutations [most often G to A, or C to T transitions (SULSTON and HODGKIN 1988)]. This procedure allowed us to rapidly localize seven mutations.

Guided by these data (not shown), we sequenced the appropriate regions of mutant DNA. For the N-terminal part of the protein including the homeodomain (exons 2–7), unambiguous sequence data were obtained for all 17 revertants. Specific point sequence changes were identified for 10 of the 17 genetically characterized HSPB revertants. Among the 17 revertants, eight carry single identified point mutations, either missense or nonsense, one carries two adjacent point mutations, while one other harbors a frame shift in the homeobox. These results are summarized in Table 3 and represented schematically in Figure 3. Identified $HSPB^-$ revertant mutations were situated in the homeodomain or in the C-terminal region. In contrast, the mutations of the $HSPB^{+/-}$ class were outside the homeodomain across the protein. The remaining class led us to identify an amino acid in the homeodomain N-terminal hinge likely to be important for functional specificity.

Class 1: pb homeotic function requires sequences both within and outside the homeodomain: Only two complete loss-of-function mutations reside in the homeodomain, among the 17 homeodomains for which we possessed unambiguous sequence data. The third lesion located in the homeodomain is a frameshift mutation generating a truncated PB^- protein whose loss of function cannot be attributed to the homeodomain *per se* (Table 3). The lone previously described revertant, $HSPB^{Rev14}$, acts as a complete loss-of-function and disrupts an evolutionarily invariant position of the homeodomain [arg53 to His; (see CRIBBS *et al.* 1995); Figure 3A). Arginine-53 has been shown to contact phosphates of the DNA backbone in both Engrailed and MATα2 homeodomains (KISSINGER *et al.* 1990; WOLBERGER *et al.* 1991). Consistent with these observations, DNA binding of the PB^{Rev14} homeodomain to a consensus target sequence *in vitro* is markedly reduced (BENASSAYAG *et al.*, 1997). This mutation thus behaves as a strong loss-of-function both *in vivo* and *in vitro*. The second mutation $HSPB^{Rev23}$ affects cysteine-27 in the non-helical central portion of the homeodomain (Figure 3A) and behaves as a complete loss-of-function *in vivo*. Taken together, these two alleles thus confirm that a point mutation in the homeodomain can abolish PB function.

Two complete loss of function mutations identified sites outside the homeodomain required for PB function. Both were located C-terminal to the homeodomain, changing Ser450 to Arg and Arg602 to Ala (Table 3 and Figure 3B), within a region containing runs of prolines or glutamines (Opa sequences). Thus a PB protein with an intact homeodomain, but carrying a

TABLE 3

Nucleotide and amino acid change associated with each HSPB revertant lines

Mutants	Phenotype	Rescue of *pb^hyp mutants*	Nucleotide position[a]	Nucleotide change[a]	Codon change	Amino acid change[a]
HSPB Rev no. 9	Class 1, HSPB$^-$	−	Beginning of exon 5	Deletion of one A (between 352 and 356)	Frameshift within the homeobox	Detectable protein
HSPB Rev no. 14	Class 1, HSPB$^-$	−	Exon 5: position 376	G→A	CGC→CAC	Arg250→His
HSPB Rev no. 23	Class 1, HSPB$^-$	−	Exon 4: position 123	T→G	TGC→GGC	Cys224→Gly
HSPB Rev no. 1	Class 1, HSPB$^-$	−	Exon 8: position 1556	A→C	AGT→CGT	Ser450→Arg
HSPB Rev no. 5	Class 1, HSPB$^-$	−	Exon 8: position 2012	A→C	GAC→GCC	Asp602→ala
HSPB Rev no. 17	Class 2, complete reversion + mild eyes reduction	−	Exon 4: position 117	C→T	CGC→TGC	Arg202→Cys
HSPB Rev no. 3	Class 2, complete reversion + eyes reduction	−	Exon 4: position 118	G→A	CGC→CAC	Arg202→His
HSPB Rev no. 2	Class 3, HSPB$^{+/-}$	+/−	Exon 9: position 158	C→T	CAG→TAG	Gln675→amb
HSPB Rev no. 19	Class 3, HSPB$^{+/-}$	+/−	Exon 2: positions 297 and 299	G→T G→T	CCA→CAA CCT→ACT	Pro67→Gln Pro68→Thr
HSPB Rev no. 20	Class 3, HSPB$^{+/-}$	+/−	Exon 6: position 754	C→A	TCC→TAC	Ser281→Tyr

[a] Refers to nucleotides and amino acid position within the *pb* sequence [Figure 3, A–D (CRIBBS *et al.* 1992].
+/−, partial rescue; −, a non-rescue.

single amino acid change well downstream of the homeodomain in the C terminal region (193 and 345 residues downstream, respectively), is no longer able to specify segmental identity.

Class 2: The amino terminus of the homeodomain is involved in PB-induced eye loss: Two additional point mutations located in the homeodomain, HSPB^{Rev3} and HSPB^{Rev17}, constitute a second class of phenotypic revertants. For HSPB^{Rev17} mild but detectable eye defects were observed following heat induction. In contrast, elevated expression of HSPB^{Rev3} protein led on occasion to a complete eye loss no longer associated with a homeotic transformation. Both lines carry a single point mutation affecting the same amino acid, changing the highly conserved arginine-5 in the homeodomain to His (HSPB^{Rev3}) or to Cys (HSPB^{Rev17}; see Table 3 and Figure 3A). These two independent revertants can detectably affect the eyes and carry a missense mutation of residue 5 of the N-terminal hinge region of the homeodomain implicated in functional specificity of homeotic proteins (LIN and MCGINNIS 1992; ZENG *et al.* 1993; CHAN and MANN 1996). Wild-type PB protein expression in the eyes can induce an eye reduction resembling that induced by expression of these revertants proteins. However, the reversion and non-rescue associated with the mutant alleles show that the lesion is not a simple loss of function. Changing Arg5 to a new identity can apparently separate explicit homeotic selector functions of the PB$^+$ protein from the eye defects. These novel properties prompted us to perform a detailed analysis of these mutations presented elsewhere (BENASSAYAG *et al.* 1997).

Class 3: Nonconserved regions outside the homeodomain contribute quantitatively to PB function: One of the most interesting aspects of the *pb* model is that partial or complete loss-of-function mutations give rise to distinct transformations (CRIBBS *et al.* 1992). Futhermore, different quantities of *pb* protein can apparently result in alternate developmental outcomes (CRIBBS *et al.* 1995). In our analysis, three revertants were found to exhibit a partial reversion of the dominant PB homeotic transformation in the antennae and in the mouthparts. These harbor point mutations outside the homeodomain, in three distinct and nonconserved regions of the protein (Figure 3B). The first, HSPB^{Rev19}, carries a double point mutation changing prolines 67 and 68 in the N-terminal portion of the protein to glutamine and threonine (Table 3). The second, HSPB^{Rev20}, harbors a point mutation changing Ser281 to Tyr (24 amino acids downstream of the homeodomain). The third mutation, HSPB^{Rev2}, creates a stop codon leading to a truncated protein lacking its C-terminal 123 amino acids. These data suggest that some C or N terminal sequences of the PB protein contribute quantitatively to normal *pb*$^+$ function.

Our data thus support the interpretation that ele-

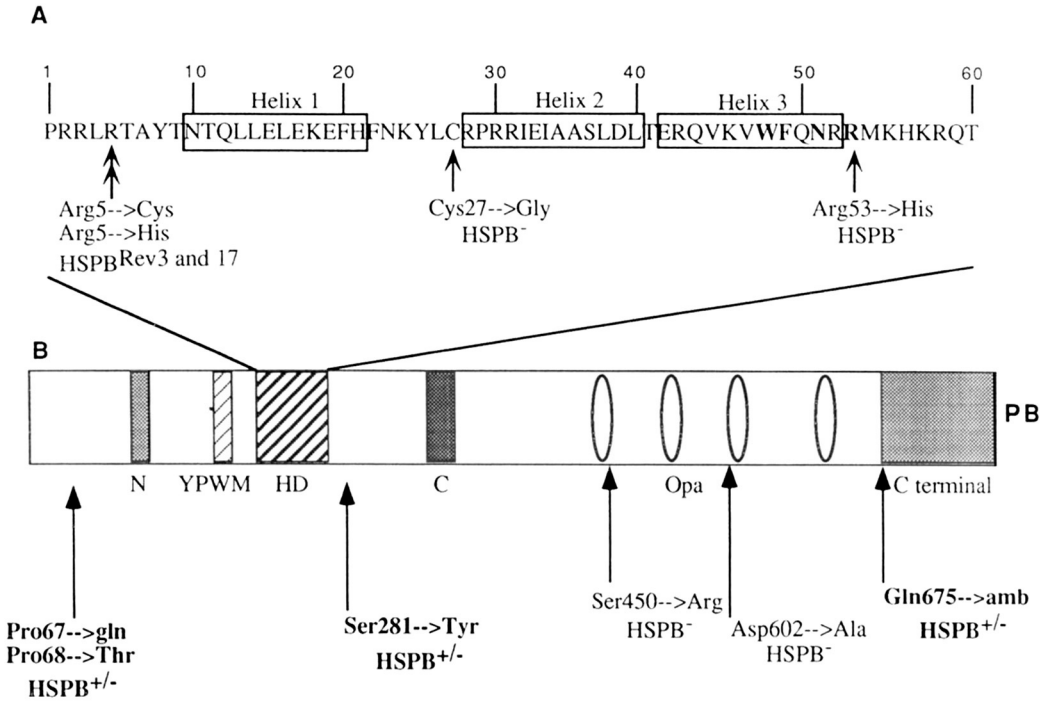


FIGURE 3.—Schematic representation of the PB protein showing the localization of identified mutations. (A) Shown at top is the sequence of the PB homeodomain. Beneath are indicated the locations of revertant homeodomain mutations (arrows) with the normal and replacing amino acids indicated. Complete loss-of-function mutations are indicated by $HSPB^-$. (B) The conserved N, YPWM, homeodomain, C motif and the C terminal domains of the PB protein are represented by boxes, and the approximate locations of Opa repeat sequences that encode runs of prolines or glutamines, by ovals (CRIBBS *et al.* 1992). Positions of sequence changes associated with the HSPB revertants are indicated beneath (arrows). Partial revertant mutations ($HSPB^{+/-}$) are distinguished by bold type.

ments, both conserved (homeodomain) and nonconserved, throughout the PB protein are required for full pb^+ function.

DISCUSSION

The present work represents the first characterization of a consequent number of point mutations altering homeotic function. The highly reproducible, dose-sensitive nature of the partial transformation induced by HSPB has permitted the isolation of a diversified collection of mutations, as revertants of a dominant phenotype. Proteins showing complete loss, partial loss or altered functions were generated and recovered *in vivo* in a homogeneous background.

We found 17 PB-expressing mutations that affect pb function in distinct ways, of which we have identified 10 lesions. The other seven mutations are closely linked to HSPB but are heat inducible and thus do not remove Hsp70 promoter function. It therefore seems probable that they are located within the pb transgene and were not detected due to technical limitations. Two obvious possibilities exist. (1) Several conserved regulatory elements are present within the intron 2 (RANDAZZO *et al.* 1991; KAPOUN and KAUFMAN 1995), whereas our analysis was limited to exonic coding sequences and thus did not examine those sequences. (2) Further, while clear sequence data were obtained for most protein-coding sequences, difficulties were often encountered in amplifying and sequencing the Opa repeat-rich regions in the C-terminal half of the PB protein.

Point mutations in the homeodomain can abolish PB function: Only four of 17 analyzed revertant alleles contain mutations localized in homeodomain-coding sequences. This result contrasts with the preponderant role attributed to the homeodomain in homeotic function. One homeodomain point mutation, located in helix 3, changes an evolutionarily invariant arginine (Figure 3B), Arg250 (or in the standard homeodomain structure Arg53 → His), that contacts the DNA backbone in co-crystals with Engrailed and MATα2 homeodomains (KISSINGER *et al.* 1990; WOLBERGER *et al.* 1991). Our results are consistent with the structural data concerning the homeodomain, since this mutation causes a strong or complete loss of function *in vivo* (CRIBBS *et al.* 1995) and reduced DNA binding affinity (BENASSAYAG *et al.* 1997). The second simple loss-of-function mutation in the homeodomain alters cysteine-224 (Cys27 → Gly) in the unstructured part of the homeodomain separating helices 1 and 2. Though less is known about this region of the homeodomain, the neighboring residue 25 makes sequence nonspecific contacts to phosphates along the DNA backbone similar to those effected by Arg53 (KISSINGER *et al.* 1990). We speculate that the strong or complete loss of PB^+ function due to the Cys27 → Gly change may result from a modified

local conformation that alters DNA-binding affinity indirectly. Combined with structural data for other homeodomains, these observations also support a crucial role for the homeodomain in homeotic function via its interactions with DNA.

A critical role for homeodomain Arg5 (N-terminal hinge) in homeodomain function: Two other homeodomain mutations identified in this screen change the same amino acid, Arginine-202 (or Arg5, in the N-terminus of the standard homeodomain). Each revertant leads to detectable adult eye defects following heat induction, though to differing extents. Several studies have shown that the N-terminal portion of the homeodomain is critical for functional specificity *in vivo* (GIBSON *et al.* 1990; LIN and MCGINNIS 1992; ZENG *et al.* 1993; EKKER *et al.* 1994; MANN 1995; CHAN and MANN 1996; ZHAO *et al.* 1996). Nonetheless, the role of the nearly invariant Arg5 in functional specificity has never been analyzed, precisely because of its very strong evolutionary conservation. It is notable that our screen, based on dominant gain-of-function phenotypes, permitted us to detect these novel properties of the mutant PB protein whereas the same mutation was viewed as a simple loss-of-function allele by the criterion of normal function in rescue experiments. A detailed analysis of these mutations is presented elsewhere (BENASSAYAG *et al.* 1997).

Quantitative contributions of sequences outside the homeodomain to PB homeotic function: The three mutations causing a partial loss of PB function are located outside the homeodomain. One is in the N-terminus of the protein, a second 25 residues downstream of the homeodomain, and a third mutation deletes the C-terminal 123 amino acids. The diminished gain-of-function and partial rescues observed could result from lessened protein stability. However, immunostaining experiments did not indicate markedly reduced accumulation in embryos following heat induction (not shown). Further, none of the three point mutations affects apparent PEST-related motifs implicated in protein stability (RECHSTEINER 1988). More interestingly, the N-terminal and/or C-terminal regions of PB may affect PB homeodomain functions within the same molecule, or interactions with other proteins necessary for full biological activity *in vivo*. Given the small reduction in activity of roughly one half detected *in vivo*, this point will no doubt be difficult to establish at a molecular level.

Implications of PB dose sensitivity: Our genetic screens were performed based on the partial homeotic transformation of antennae to maxillary palps induced by a single $HSPB^+$ element. Two copies of the same element completely transforms antennae to palps. Flies carrying three $HSPB^+$ copies die. This lethality is at the level of the organism, since clones of marked cells carrying three $HSPB^+$ elements can be generated by mitotic recombination (not shown). One partial revertant copy ($HSPB^{+/-}$) can be considered equivalent to 0.5 wild-type copies (since two $HSPB^{+/-}$ copies are phenotypically similar to one wild-type copy). The difference between levels of PB activity that are phenotypically undetectable and overtly lethal is thus only a factor of about six (0.5 to three copies). Normal regulation of *pb* activity probably must respect a narrow window of acceptable expression levels. These levels might be important, for example, for combinatorial interactions with other transcription factors. Such considerations may be important for normal *pb* functions, for example in the labial palps where *pb* acts in concert with *Scr* to generate labial rather than prothoracic or maxillary identities (CRIBBS *et al.* 1995). Ultimately, to understand the biological logic of homeotic function it will be necessary to comprehend how these small differences are employed in a coherent way.

Role of the C-terminal region of PB protein: Mutations with different developmental effects alter the C-terminal half of the PB protein. Two alleles, one a missense mutation 24 residues downstream of the homeodomain and the other a nonsense mutation that truncates the last 123 amino acids, show similar partial loss of PB^+ function. The latter is consistent with previous results showing that loss of PB epitopes in exon 9 (the C-terminal 163 amino acids) can result in partial loss of pb^+ function (CRIBBS *et al.* 1992). The separation in the linear protein sequence of these mutations with similar effects might reflect genuine interactions of dispersed elements that are reassembled in the folded protein.

In marked contrast to the partial loss-of-function alleles, two point missense mutations (located ~200 and 350 amino acids downstream of the homeodomain) show complete loss of PB function. Neither mutation resides within an evolutionarily conserved motif, clearly supporting the value of an approach based on function such as the present screen, rather than one guided primarily by structural conservation, in studying homeotic/Hox activity.

Sequences C-terminal to the homeodomain (the C-tail) were found to influence UBX function *in vivo*, playing an important role in determining UBX specificity without affecting homeodomain DNA binding (CHAN and MANN 1993). Similarly, sequences C-terminal of the yeast MATα2 homeodomain are important for interactions with the MATa1 homeodomain and for target gene selection *in vivo* (MAK and JOHNSON 1993; STARK and JOHNSON 1994). The partial revertant 24 residues downstream of the PB homeodomain may be comparable to the loss of the UBX C tail (CHAN and MANN 1993). Our results with PB extend the previous study, since simple point mutations of the nonconserved C-terminal region abolish any detectable PB homeotic function. We conclude that sequences downstream of the homeodomain are necessary for normal PB function, as previously deduced for UBX. However,

it is not clear to what extent previous observations for UBX are comparable to PB since the C-tails differ not only in sequence but are also of markedly different sizes (35 amino acids for UBX *vs.* 640 for PB). If, as for UBX, the C-tail plays a role independent of obvious DNA binding functions, it will be of interest to examine potential protein-protein interactions influencing developmental outcome.

Evolutionarily conserved protein motifs and requirements for *pb* function: The PB protein exhibits striking similarity to its mammalian homologues Hox-A2 and Hox-B2, in the homeodomain as well as several regions outside the homeodomain including the highly conserved Tyr-Pro-Trp-Met (YPWM) motif present in most homeotic/Hox proteins (CRIBBS *et al.* 1992). Apart from the homeodomain, no identified mutation touches any of the three other strongly conserved sequence elements present in vertebrate Hox-A2/B2 proteins. Given the small size of these elements with respect to the 798 residue PB protein, this point is perhaps not surprising. Still, the high evolutionary conservation of such elements is considered as strong evidence for their important roles in homeoprotein function and a guide for site-directed mutagenesis to test function. Our data lead us to conclude that the apparently nonconserved remainder of the protein also plays a critical role. One way in which this might occur is in the correct folding of the conserved protein elements. Mutations affecting positioning of conserved elements could act as strongly as those affecting the conserved motifs themselves. These mutations might also affect sites governing species-specific rather than general HOM functions. Further studies like the one presented here should help to elucidate the nature of homeotic protein function central to proper development.

We thank STÉPHANE NOSELLI, BRUNO GLISE, ALAIN VINCENT and HENRI-MARC BOURBON for their critical readings of the manuscript. C.B., M.B. and L.S. were supported by graduate fellowships from the Ministère de l'Enseignement Supérieur et de la Recherche (MESR); M.B. and L.S. also benefited from graduate training fellowships from the Association pour la Recherche contre le Cancer (ARC). This work was made possible by ongoing funding from the Centre National de Recherche Scientifique and grants from the ARC and the MESR.

LITERATURE CITED

ANANTHAN, J., R. BALER, D. MORRISSEY, J. ZUO, Y. LAN *et al.*, 1993 Synergistic activation of transcription is mediated by the N-terminal domain of Drosophila fushi tarazu homeoprotein and can occur without DNA binding by the protein. Mol. Cell. Biol. **13:** 1599–1609.

ANDREW, D. J., and M. P. SCOTT, 1992 Downstream of the homeotic genes. New Biol. **4:** 5–15.

BEACHY, P. A., J. VARKEY, K. E. YOUNG, D. P. VON KESSLER, B. I. SUN *et al.*, 1993 Cooperative binding of an Ultrabithorax homeodomain protein to nearby and distant DNA sites. Mol. Cell. Biol. **13:** 6941–6956.

BENASSAYAG, C., L. SEROUDE, M. BOUBE, M. ERARD and D.L. CRIBBS, 1997 A homeodomain point mutation of the Drosophila proboscipedia protein provoues eye loss independently of homeotic function. Mech. Dev. (in press).

BOTAS, J., 1993 Control of morphogenesis and differentiation by HOM/Hox genes. Curr. Opin. Cell. Biol. **5:** 1015–1022.

CAPOVILLA, M., M. BRANDT and J. BOTAS, 1994 Direct regulation of decapentaplegic by Ultrabithorax and its role in Drosophila midgut morphogenesis. Cell **76:** 461–475.

CHAN, S. K., and R. S. MANN, 1993 The segment identity functions of Ultrabithorax are contained within its homeo domain and carboxy-terminal sequences. Genes Dev. **7:** 796–811.

CHAN, S. K., and R. S. MANN, 1996 A structural model for a homeotic protein-extradenticle-DNA complex accounts for the choice of HOX protein in the heterodimer. Proc. Natl. Acad. Sci. USA **93:** 5223–5228.

CHAN, S. K., L. JAFFE, M. CAPOVILLA, J. BOTAS and R. S. MANN, 1994 The DNA binding specificity of Ultrabithorax is modulated by cooperative interactions with extradenticle, another homeoprotein. Cell **78:** 603–615.

COPELAND, J. W., A. NASIADKA, B. H. DIETRICH and H. M. KRAUSE, 1996 Patterning of the Drosophila embryo by a homeodomain-deleted Ftz polypeptide. Nature **379:** 162–165.

CRIBBS, D. L., M. A. PULTZ, D. JOHNSON, M. MAZZULLA and T. C. KAUFMAN, 1992 Structural complexity and evolutionary conservation of the Drosophila homeotic gene proboscipedia. EMBO J. **11:** 1437–49.

CRIBBS, D. L., C. BENASSAYAG, F. M. RANDAZZO and T. C. KAUFMAN, 1995 Levels of homeotic protein function can determine developmental identity: evidence from low level expression of the Drosophila homeotic gene proboscipedia under Hsp70 control. EMBO J. **14:** 767–778.

EKKER, S. C., K. E. YOUNG, D. P. VON KESSLER and P. A. BEACHY, 1991 Optimal DNA sequence recognition by the Ultrabithorax homeodomain of Drosophila. EMBO J. **10:** 1179–1186.

EKKER, S. C., D. P. VON KESSLER and P. A. BEACHY, 1992 Differential DNA sequence recognition is a determinant of specificity in homeotic gene action. EMBO J. **11:** 4059–4072.

EKKER, S. C., D. G. JACKSON, D. P. VON KESSLER, B. I. SUN, K. E. YOUNG *et al.*, 1994 The degree of variation in DNA sequence recognition among four Drosophila homeotic proteins. EMBO J. **13:** 3551–3560.

FITZPATRICK, V. D., S. A. PERCIVAL, C. J. INGLES and H. M. KRAUSE, 1992 Homeodomain-independent activity of the fushi tarazu polypeptide in Drosophila embryos. Nature **356:** 610–612.

GARCIA-BELLIDO, A., 1975 Genetic control of wing disc development in Drosophila. Ciba Foundation Symp. **29:** 161–182.

GARCIA-BELLIDO, A., 1977 Homeotic and atavic mutations in insects. Am. Zool. **17:** 613–629.

GEHRING, W. J., M. MÜLLER, M. AFFOLTER, A. PERCIVAL-SMITH, M. BILLETER *et al.*, 1990 The structure of the homeodomain and its functional implications. Trends Genet. **6:** 323–329.

GIBSON, G., A. SCHIER, P. LEMOTTE and W. J. GEHRING, 1990 The specificities of Sex combs reduced and Antennapedia are defined by a distinct portion of each protein that includes the homeodomain. Cell **62:** 1087–1103.

GONZALEZ-CRESPO, S., and G. MORATA, 1995 Control of Drosophila adult pattern by extradenticle. Development **121:** 2117–2125.

GONZALEZ-REYES, A., A. MACIAS and G. MORATA, 1992 Autocatalysis and phenotypic expression of Drosophila homeotic gene Deformed: its dependence on polarity and homeotic gene function. Development **116:** 1059–1068.

GOULD, A. P., and R. A. H. WHITE, 1992 *Connectin*, a target of homeotic gene control in Drosophila. Development **116:** 1163–1174.

HALDER, G., P. CALLAERTS and W. J. GEHRING, 1995 Induction of ectopic eyes by targeted expression of the *eyeless* gene in Drosophila. Science **267:** 1788–1792.

HAYASHI, S., and M. P. SCOTT, 1990 What determines the specificity of action of Drosophila homeodomain proteins? Cell **63:** 883–894.

HEUER, J. G., and T. C. KAUFMAN, 1992 Homeotic genes have specific functional roles in the establishment of the Drosophila embryonic peripheral nervous system. Development **115:** 35–47.

HEUER, J. G., K. LI and T. C. KAUFMAN, 1995 The Drosophila homeotic target gene *centrosomin* (*cnn*) encodes a novel centrosomal protein with leucine zippers and maps to a genomic region required for midgut morphogenesis. Development **121:** 3861–3876.

JOHNSON, F. B., E. PARKER and M. A. KRASNOW, 1995 Extradenticle protein is a selective cofactor for the Drosophila homeotics: role

of the homeodomain and YPWM amino acid motif in the interaction. Proc. Natl. Acad. Sci. USA **92:** 739–743.

KAPOUN, A. M., and T. C. KAUFMAN, 1995 A functional analysis of 5′, intronic and promoter regions of the homeotic gene *proboscipedia* in *Drosophila melanogaster.* Development **121:** 2127–2141.

KAUFMAN, T. C., 1978 Cytogenetic analysis of chromosome 3 in *Drosophila melanogaster:* isolation and characterization of four new alleles of the *proboscipedia* (*pb*) locus. Genetics **90:** 579–596.

KAUFMAN, T. C., M. SEEGER and G. OLSEN, 1990 Molecular and genetic organization of the *Antennapedia* gene complex of *Drosophila melanogaster.* Adv. Genet. **27:** 309–362.

KENYON, C., 1994 If birds can fly, why can't we? Homeotic genes and evolution. Cell **78:** 175–180.

KISSINGER, C. R., B. S. LIU, B. E. MARTIN, T. B. KORNBERG and C. O. PABO, 1990 Crystal structure of an engrailed homeodomain-DNA complex at 2.8 Å resolution: a framework for understanding homeodomain-DNA interactions. Cell **63:** 579–590.

KUZIORA, M. A., and W. MCGINNIS, 1988 Autoregulation of a Drosophila homeotic selector gene. Cell **55:** 477–485.

LAMKA, M. L., A. M. BOULET and S. SAKONJU, 1992 Ectopic expression of UBX and ABD-B proteins during Drosophila embryogenesis: competition, not a functional hierarchy, explains phenotypic suppression. Development **116:** 841–854.

LEWIS, E. B., 1978 A gene complex controlling segmentation in Drosophila. Nature **276:** 565–570.

LEWIS, E. B., and F. BACHER, 1968 Method of feeding ethyl methanesulphonate to *Drosophila* males. Dros. Inf. Serv. **43:** 193–194.

LI, K., and T. C. KAUFMAN, 1996 The homeotic target gene *centrosomin* encodes an essential centrosomal component. Cell **85:** 585–596.

LIN, L., and W. MCGINNIS, 1992 Mapping functional specificity in the Dfd and Ubx homeo domains. Genes Dev. **6:** 1071–1081.

MAK, A., and A. D. JOHNSON, 1993 The carboxy-terminal tail of the homeo domain protein alpha 2 is required for function with a second homeo domain protein. Genes Dev. **7:** 1862–1870.

MALICKI, J., K. SCHUGHART and W. MCGINNIS, 1990 Mouse Hox-2.2 specifies thoracic segmental identity in Drosophila embryos and larvae. Cell **63:** 961–967.

MANN, R. S., 1995 The specificity of homeotic gene function. Bioessays **17:** 855–863.

MANN, R. S., and D. S. HOGNESS, 1990 Functional dissection of Ultrabithorax proteins in *D. melanogaster.* Cell **60:** 597–610.

MCCABE, P. C., 1990 Production of single-stranded DNA by asymmetric PCR, pp. 76–83 in *PCR Protocols,* edited by M. A. INNIS, D. H. GELFAND, J. J. SNINSKY and T. J. WHITE. Academic Press, San Diego, CA.

MCGINNIS, W., and R. KRUMLAUF, 1992 Homeobox genes and axial patterning. Cell **68:** 283–302.

MONTANDON, A. J., P. M. GREEN, F. GIANNELLI and D. R. BENTLEY, 1989 Direct detection of point mutations by mismatch analysis: application to haemophilia B. Nucleic Acids Res. **17:** 3347–3358.

NOSE, A., V. B. MAHAJAN and C. S. GOODMAN, 1992 Connectin: a homophilic cell adhesion molecule expressed on a subset of muscles and the motoneurons that innervate them in Drosophila. Cell **70:** 553–567.

O'HARA, E., B. COHEN, S. M. COHEN and W. MCGINNIS, 1993 *Distal-less* is a downstream gene of *Deformed* required for ventral maxillary identity. Development **117:** 847–856.

PEIFER, M., and E. WIESCHAUS, 1990 Mutations in the Drosophila gene *extradenticle* affect the way specific homeo domain proteins regulate segmental identity. Genes Dev. **4:** 1209–1223.

PEIFER, M., F. KARCH and W. BENDER, 1987 The bithorax complex: control of segmental identity. Genes Dev. **1:** 891–898.

PULTZ, M. A., R. J. DIEDERICH, D. L. CRIBBS and T. C. KAUFMAN, 1988 The *proboscipedia* locus of the Antennapedia complex: a molecular and genetic analysis. Genes Dev. **2:** 901–920.

RANDAZZO, F. M., D. L. CRIBBS and T. C. KAUFMAN, 1991 Rescue and regulation of *proboscipedia*: a homeotic gene of the Antennapedia Complex. Development **113:** 257–271.

RAUSKOLB, C., M. PEIFER and E. WIESCHAUS, 1993 *extradenticle,* a regulator of homeotic gene activity, is a homolog of the homeobox-containing human proto-oncogene *pbx1.* Cell **74:** 1101–1112.

RAUSKOLB, C., K. M. SMITH, M. PEIFER and E. WIESCHAUS, 1995 *extradenticle* determines segmental identities throughout Drosophila development. Development **121:** 3663–3673.

RECHSTEINER, M., 1988 Regulation of enzyme levels by proteolysis: the role of PEST regions. Adv. Enzyme Reg. **27:** 135–151.

SCHIER, A. F., and W. J. GEHRING, 1992 Direct homeodomain-DNA interaction in the autoregulation of the *fushi tarazu* gene. Nature **356:** 804–807.

SCHNEUWLY, S., R. KLEMENZ and W. J. GEHRING, 1987 Redesigning the body plan of Drosophila by ectopic expression of the homoeotic gene *Antennapedia.* Nature **325:** 816–818.

STARK, M. R., and A. D. JOHNSON, 1994 Interaction between two homeodomain proteins is specified by a short C-terminal tail. Nature **371:** 429–432.

SULSTON, J., and J. HODGKIN, 1988 Methods, pp. 587–606 in *The Nematode Caenorhabditis elegans,* edited by W. B. WOOD. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

VACHON, G., B. COHEN, C. PFEIFLE, M. E. MCGUFFIN, J. BOTAS *et al.,* 1992 Homeotic genes of the Bithorax complex repress limb development in the abdomen of the Drosophila embryo through the target gene *Distal-less.* Cell **71:** 437–450.

VAN DIJK, M., and C. MURRE, 1994 *extradenticle* raises the DNA binding specificity of homeotic selector gene products. Cell **78:** 617–624.

WOLBERGER, C., A. K. VERSHON, B. LIU, A. D. JOHNSON and C. O. PABO, 1991 Crystal structure of a MATα2 homeodomain-operator complex suggests a general model for homeodomain-DNA interactions. Cell **67:** 517–528.

ZENG, W., D. J. ANDREW, L. D. MATHIES, M. A. HORNER and M. P. SCOTT, 1993 Ectopic expression and function of the *Antp* and *Scr* homeotic genes: the N terminus of the homeodomain is critical to functional specificity. Development **118:** 339–352.

ZHAO, J. J., R. A. LAZZARINI and L. PICK, 1996 Functional dissection of the mouse *Hox-a5* gene. EMBO J. **15:** 1313–1322.

Communicating editor: T. SCHÜPBACH